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Whole Cell Lysate Enzyme Immunoassays vs. Recombinant Glycoprotein G2-Based Immunoassays for HSV-2 Seroprevalence Studies

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Seroepidemiology studies of herpes simplex virus type 2 (HSV-2) infections have been difficult to carry out because antibodies to HSV type 1 (HSV-1) show an extensive cross-reactivity with HSV-2 antigens. Many kits available currently are not entirely type specific for serodiagnosis of HSV-2 infections and therefore do not allow reliable discrimination of past exposure to these closely related alphaherpes viruses. Attempts to develop type-specific antigens have focused on the envelope glycoproteins, particularly glycoprotein G (gG). A cross-sectional study was carried out to examine the seroprevalence of antibodies to HSV-2 among healthy university students, using different methods: a whole cell lysate enzyme-linked immunosorbent assay (ELISA), two different ELISAs, and a newly developed immunoblot assay, the last three based on recombinant gG2. HSV-2 prevalence was 24 times higher with the whole cell lysate ELISA (31%; 95% confidence interval [CI]: 27–35%) than the ELISAs and the immunoblot assay based on recombinant gG2 (1.3%; 95% CI: 0.1–2.5%), thus showing the inaccuracy of commercial tests based on whole-antigen preparations for epidemiological studies. Laboratories should be cautious and ensure that commercial tests for HSV typing are based on type-specific glycoproteins. ***J. Med. Virol. 59:502–506, 1999.***

KEY WORDS: herpes simplex type 2 virus; serology; laboratory diagnosis; enzyme immunoassays; epidemiology

INTRODUCTION

The identification of two subtypes of herpes simplex virus (HSV) has led to the development of serological methods, in particular enzyme immunoassays, with the aim of discriminating type-specific HSV antibodies. Some commercial tests available currently are of limited value because they are not entirely type specific for differentiating HSV-1 and HSV-2 antibodies [Mertz, 1993; Kinghorn, 1994]. As stated by several investigators [Johnson et al., 1989; Gibson et al., 1990], these limitations have made seroepidemiology studies of HSV-2 difficult.

Commercial enzyme immunoassays have two important advantages: their low cost and widespread availability. These tests are able to distinguish subjects who are seropositive for herpes simplex from subjects who are seronegative [Gilman and Docherty, 1977; Coleman et al., 1983; Ashley et al., 1991], but provide adequate sensitivity and specificity to detect HSV antibodies only when information regarding the virus type is not required, for example, when screening patients who are to receive chemotherapy or organ transplantation to establish the need of antiviral prophylaxis or for detecting seroconversion in primary infections [Mertz, 1993].

The inability of commercial assays based on whole-antigen preparations to discriminate HSV type-specific antibodies is due to the high level of HSV common antigens present among subtypes, giving rise to antibod-

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ies that cross-react [McClung et al., 1983; Ashley and Militoni, 1987]. Attempts to develop type-specific antigens have focused on the envelope glycoproteins, which are major immunogens and have important biological functions. Only a few truly type-specific glycoproteins have been identified so far. Glycoprotein G (gG) has been used for the development of assays for HSV subtyping [Lee et al., 1985; Ashley et al., 1988; Nahmias et al., 1990; Ho et al., 1992] on the basis that there is very limited sequence homology between gG1 and gG2. As these tests require large quantities of glycoprotein that are difficult to obtain, assays based on recombinant proteins [Sánchez-Martínez et al., 1991] may be helpful. Western blotting [Ashley et al., 1988], although relatively difficult to interpret in certain cases, such as HSV-1 and HSV-2 coinfection, is still considered the "gold standard" for HSV typing. It is, however, a sophisticated and expensive assay for large-scale studies, not easily adaptable to commercial laboratory use and, therefore, not adequate as a first-line screening test. HSV-2 tests suitable for seroepidemiological studies should be cheap, simple to use, highly sensitive and accurate for identifying subjects with antibodies to HSV-2, and able to detect low levels of antibodies. Newly developed immunoassays based on HSV type-specific recombinant glycoproteins, such as gG2, meet these requirements.

Due to the unacceptably high rates of false-positive results regarding past HSV-2 infection provided by many commercially available serological assays and the misleading results obtained in the past when conducting epidemiological surveys [Cowan et al., 1994], a cross-sectional study was undertaken to examine the seroprevalence of antibodies to HSV-2 among healthy university students, using different serological methods based on whole-antigen preparations or recombinant gG2.

METHODS

Subjects

The required sample size calculation was based on the following assumptions: an anticipated prevalence of HSV-2 antibodies was assumed conservatively to be 12% in the age range 18–25 years [Cowan et al., 1994]; an absolute precision of the results of 4 percentage points; and a confidence level of 95%. To fulfil these requirements, a total of 306 subjects were required. Subjects were recruited to the study from students attending the School of Medicine and the School of Nursing of two Spanish universities: "Complutense" (Madrid) and "Autónoma" (Barcelona). The age of the students ranged from 18 to 25 years (mean age, 23 years; SD 1.2). One hundred eighty-five (60%) subjects were women and 121 (40%) were men.

The study protocol was approved by the Ethical Review Committee of the San Carlos Hospital, Madrid. Written informed consent was obtained from all the subjects participating in the study before enrolment.

Procedure

All serum samples were screened for HSV-2 IgG antibodies by two different enzyme-linked immunosorbent assays (ELISA): a whole-cell lysate ELISA (ELISA 1) and a second ELISA based on type-specific gG2 (ELISA 2). Positive sera were then re-evaluated for HSV-1 IgG and HSV-2 IgG antibodies by a third ELISA (ELISA 3) and a newly developed immunoblot assay, both based on type-specific gG1 and gG2, and a whole cell lysate Western blot. All samples were tested in a coded fashion by the laboratory staff. All tests were carried out on the same serum sample from each subject without knowledge of the serological results of the other assay.

ELISA 1. In the ELISA 1 assay (Menarini Diagnostics, Barcelona, Spain), HSV-2 antigen was prepared by glycine extraction of Vero cells infected with HSV-2 strain G (ATCC), purified to reduce host cell components and ultraviolet inactivated. Test samples were diluted 1:20 and controls allowed to react with the microtiter wells coated with the antigen. If there are specific antibodies, they will bind to the well surface. After a 20-min incubation at room temperature, the wells were washed to remove unbound material. In a second step, the anti-human IgG/horseradish peroxidase conjugate was added to the wells, resulting in the specific antibody sandwiched between the solid phase antigen and enzyme conjugate antibody. After a second incubation period, the wells were washed to remove unbound material and a substrate/chromogen solution was added and incubated. The enzyme reaction generated a coloured product that was measured photometrically. The optical density is proportional to the amount of anti-HSV-2 antibody present in the serum specimen.

ELISA 2. The ELISA 2 assay (Centocor Inc, Malvern, PA) used polystyrene microwells coated with recombinant type 2 antigen (modified gG-2) not shared by HSV type 1. The recombinant antigen is produced in a baculovirus expression system that minimises nonspecific reactions and favours retention of the natural antigenic characteristics. Briefly, serum was diluted 1:20 and incubated for 30 min at room temperature in microwells coated with recombinant gG2. The bound IgG-class antibodies were then incubated with an anti-human IgG/horseradish peroxidase conjugate. Bound conjugate was detected by incubating with enzyme substrate and tetramethylbenzidine (TMB) as a chromogen. Sensitivity and specificity of the test were found to be 95% and 92%, respectively [García-Corbeira et al., 1998].

ELISA 3. The ELISA 3 assay (MRL Diagnostics, Cypress, CA) detects HSV-type-specific IgG. Briefly, serum was diluted 1:100 and incubated for 1 hr at room temperature in microwells coated with either recombinant gG1 or recombinant gG2. Peroxidase-labelled goat anti-human IgG was then added and TMB was used as the chromogen. The recombinant gG1 and gG2 antigens were both baculovirus derived and affinity puri-

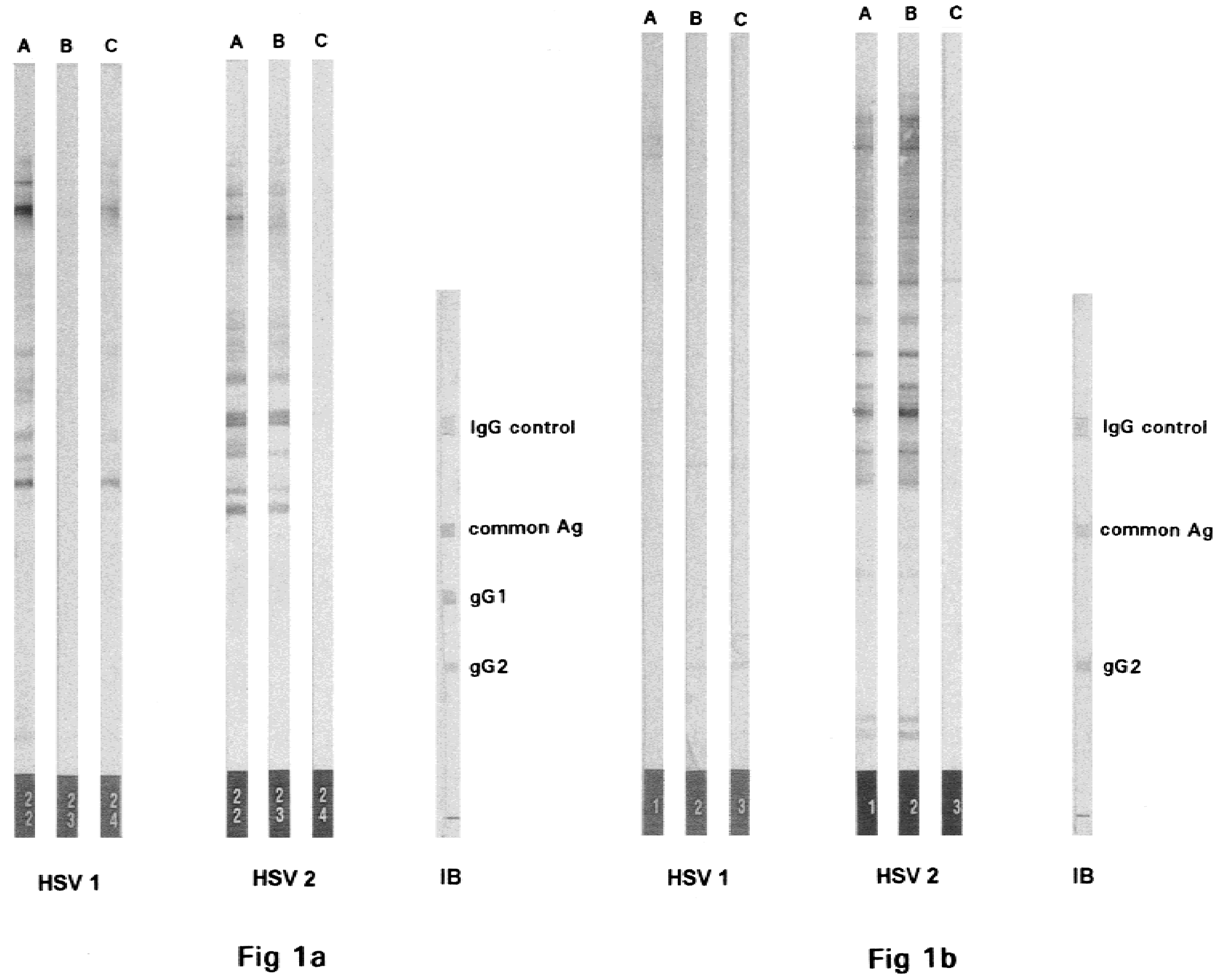

Fig. 1. **a:** Typical pattern of a sample positive for both HSV-1- and HSV-2-positive antibodies by Western blot and immunoblot. **b:** Typical pattern of a sample negative for HSV-1 antibodies and positive for HSV-2 antibodies by Western blot and immunoblot.

fied. Sensitivity and specificity of the test were found to be 98% and 91%, respectively [García-Corbeira et al., 1998].

Immunoblot assay. This assay (MRL Diagnostics, Cypress, CA) was used as directed by the manufacturers. Serum was diluted 1:100 and incubated at room temperature with nitrocellulose membranes containing an HSV common antigen, gG1 and gG2 applied individually to the membrane (Fig. 1). After washing the membrane, alkaline phosphatase-labelled goat anti-human IgG was added and incubated for 30 min. The presence of HSV antibodies was detected using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT). The sample was considered positive when both the HSV-type-common antigen and the HSV-type-specific antigen were reactive. The HSV-type-common band reacts with all HSV-positive sera and is composed of a cocktail of HSV-1- and HSV-2-type-common antigens. Both the gG1 and gG2 antigens are recombinant proteins produced in the baculovirus expression system and affinity purified before application to the membrane. Sensitivity and specificity were found to be 98% and 95%, respectively [García-Corbeira et al., 1998].

Western blot. The Western blot (MRL Diagnostics) assay was modified from Ashley et al. [1988]. Briefly, HSV-1 (strain MacIntyre) and HSV-2 (strain MS) were grown in Vero cells and, when cytopathic effect was present in 90–100% of the cells, the cultures were harvested. The HSV-infected Vero cells were washed and lysed in phosphate-buffered saline buffer containing 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100, 1.0% deoxycholic acid, and 1 mM PMSF. The viral lysates were used for both nitrocellulose Western blot strip production (10% SDS- polyacrylamide gel electrophoresis [PAGE] gels) and as antibody absorption diluents. Serum samples were prediluted in Tris/PMS buffer, HSV-1 and HSV-2 nitrocellulose strips for 1 hr at room temperature. The Western blot strips were then washed and alkaline phosphatase-labelled goat antihuman IgG (Jackson Immunoresearch, West Grove, PA) added. The presence of HSV antibodies was determined using BCIP/NBT (Kirkegaard and Perry, Gaithersburg, MD). The presence of HSV-1 and HSV-2

TABLE I. Detection of HSV-2 and HSV-1 Antibodies in a Panel of 94 Sera Classified Previously as HSV-2 Positive by a Viral Lysate-Based ELISA

	ELISA 3	Immunoblot	Western blot
HSV-2 negative/HSV-1 positive	86	86	87
HSV-2 positive/HSV-1 positive	4	4	4
HSV-2 negative/HSV-1 negative	4	4	3
Total	94	94	94

HSV, herpes simplex virus; ELISA, enzyme-linked immunosorbent assay.

specific antibody was determined by detection of type-specific antibody reactivity, which was eliminated when the appropriate type-specific antigen was used as a sorbent (see Fig. 1).

Figure 1 shows the typical patterns of a sample positive for both HSV-1 and HSV-2 antibodies (Fig. 1a) and of a sample negative for HSV-1 antibodies and positive for HSV-2 antibodies (Fig. 1b) by Western blot and immunoblot assay. The strips labelled "HSV-1" are HSV-1 antigen Western blot strips and those labelled "HSV-2" are HSV-2 antigen strips. Each strip is labelled as A, B, or C: the "A" strip was the sera diluted in sample buffer only, in "B" strips the sera were absorbed with HSV-1 antigen before being added to the Western blot strips, and in "C" strips the sera were absorbed with HSV-2 antigen before being added to the Western blot strips.

Figure 1a shows that the serum reacts with HSV-1 antigen (HSV-1A). The HSV-1 reactivity is removed when the serum is absorbed with HSV-1 antigen (HSV-1B). However, HSV-1 reactivity remains when the serum is absorbed with HSV-2 antigen (HSV-1C). The sample is also reactive with HSV-2 (HSV-2A) and remains reactive with HSV-2 when absorbed with HSV-1 antigen (HSV-2B). This Western blot pattern is typical of a sample positive for both HSV-1 and HSV-2 antibodies. The immunoblot strip demonstrates IgG reactivity to HSV common antigen gG1 and gG2 recombinant proteins.

Figure 1b shows weak reactivity to HSV-1 with the HSV-1A strip. This reactivity is removed by absorption with HSV-1 antigen (HSV-1B) and HSV-2 antigen (HSV-1C). Strong reactivity is present to HSV-2 (HSV-2A), which is not affected by HSV-1 absorption (HSV-2B); however, the HSV-2 reactivity is removed completely by absorption with HSV-2 antigen (HSV-2C). This pattern is typical of a sample negative for HSV-1 antibody and positive for HSV-2 antibodies. The immunoblot strip demonstrated reactivity to the HSV common antigen and gG2 recombinant protein but no reactivity to gG1. This immunoblot pattern is considered as HSV-1 negative and HSV-2 positive.

RESULTS

Ninety-five (57 women and 38 men) of the 306 sera tested were found to be positive on the whole cell lysate ELISA (ELISA 1), whereas with the recombinant ELISA (ELISA 2), HSV-2 antibodies were detected in only 4 sera (3 women and 1 man), the remaining 302 sera were negative. To explain this discrepancy, all except one of the specimens found to be positive with the first assay were tested by another recombinant ELISA (ELISA 3) and a rapid immunoblot assay. The 94 specimens were also tested by the Western blot as the reference technique. There was insufficient serum for the additional tests in one case. Results are summarised in Table I. Only the 4 positive samples by ELISA 2 were confirmed as positive for HSV-2 and 90 specimens were found to be negative for HSV-2 by ELISA 3 as well as by immunoblot and Western blot. Eighty-seven of the sera identified as HSV-2 negative by the Western blot assay were found to be positive for HSV-1, indicating that they were classified erroneously as HSV-2 positive by the whole cell lysate ELISA (ELISA 1) due to the cross-reactivity exhibited by antibodies to HSV-1. Three sera were negative for both HSV-1 and HSV-2 and were classified incorrectly as HSV-2 positive by ELISA 1 probably due to cross-reactivity with other *Herpetoviridae*. Finally, ELISA 3 and immunoblot assay failed to identify one sample as HSV-1 positive when compared with the reference technique (whole cell lysate Western blot), but it must be noted that Western blot specificity was achieved by the absorption of sera with HSV-1 and HSV-2 antigens.

Due to the high cross-reactivity between HSV-1 antibodies and HSV-2 antigens, HSV-2 prevalence found in the sample of university students was 24 times higher with the whole cell lysate ELISA (31%; 95%: CI: 27–35%) than with the ELISAs and the immunoblot assay based on recombinant gG2 (1.3%; 95%: CI: 0.1–2.5%), thus showing the inaccuracy of commercial tests based on whole-antigen preparations for epidemiological studies. Both recombinant ELISAs (ELISA 2 and 3) and the immunoblot assay provided accurate results when compared with the Western blot assay, minimising HSV-1 antibodies cross-reactivity with HSV-2 antigens. The immunoblot assay eliminates the highly technical procedures and interpretation involved with the use of whole viral lysate blots and absorption of sera with HSV-1 and HSV-2 antigens.

DISCUSSION

New tests able to detect type-specific gG from HSV-2 were first described in 1985 [Lee et al., 1985]. Since then, several enzyme immunoassays based on either native purified or recombinant gG2 have been developed [Sánchez-Martínez et al., 1991; Ho et al., 1992]. These assays were marketed primarily for diagnosis of HSV-2 infections rather than for use in seroepidemiology. Few surveys describing the seroepidemiology of HSV-2 in European countries using type-specific tests

have been published so far. Seroprevalence rates of 33%, 17%, 10%, and 8% have been reported in pregnant women from Sweden [Forsgren et al., 1994], France [Nahmias et al., 1990], the UK [Ades et al., 1989], and Italy [Nahmias et al., 1990], 8% in British blood donors [Cowan et al., 1994] and 4% in the Spanish general population [García-Corbeira et al., 1999]. In these studies, different serologic assays were used: ELISAs based on gG2 obtained by affinity purification with a lectin [Ades et al., 1989; Forsgren et al., 1994] or with monoclonal antibodies [Nahmias et al., 1990], recombinant gG2 [García-Corbeira et al., 1999] and Western blotting [Cowan et al., 1994]. Sensitivity and specificity of the tests may vary, making results difficult to compare.

Type-specific assays based on recombinant gG2 may have wide application for HSV-2 seroepidemiological surveys. Cost and simplicity should be considered when choosing between the different techniques and formats. Because commercial tests based on whole-antigen preparations are likely to remain on the market for years, laboratories should be cautious and ensure that commercial tests for HSV typing are based on type-specific glycoproteins.

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